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Award Number: DAMD17-02-1-0261

TITLE: Nuclear Imaging for Assessment of Prostate Cancer Gene Therapy

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REPORT DATE: April 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-04-2006	**2. REPORT TYPE** Annual	**3. DATES COVERED** *(From - To)* 12 Mar 2005 – 11 Feb 2006
4. TITLE AND SUBTITLE Nuclear Imaging for Assessment of Prostate Cancer Gene Therapy		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** DAMD17-02-1-0261 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Dongfeng Pan, Ph.D. E-mail: dp3r@virginia.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia Charlottesville, VA 22908-1339		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Combination of the cytotoxic viral thymidine kinase (tk) and the prodrug, acyclovir (ACV) has been reported to inhibit the growth of the C4-2 tumor, a subline of LNCaP. However, it remains unsolved to non-invasively detect the in vivo distribution, expression and persistence of the toxic gene as well as to evaluate the therapeutic effect. In this project, we will develop a nuclear gene imaging approach to assist the cytotoxic gene therapy study for prostate cancer. Objective/Hypothesis: The distribution, expression, and persistence of the prostate specific Ad-PSA-tk in the C4-2 tumor xenograft model will be non-invasively and repeatedly determined in vivo by tracing the radiolabeled TK substrates with a SPECT imaging modality. Specific Aim of the first year: To synthesize a radiolabeled TK substrate, 2'-Deoxy-2'fluoro-5-{3-oxo[N,N-bis(2-mercaptoethyl)ethylenediaminato][Tc-99m] technetium(V)-1(E)-propenyl}uridine, for TK detection using a small animal gamma detector. Progress and outcome: In last report of 2003 which covers from September of 2002 to March of 2003, we reported our efforts to synthesize fragments A and B. In this report we successfully linked the radiometal chelator with fluorothymidine. We will characterize the structure of the final tracer and test the pharmacokinetics and pharmacodynamics of the tracer in next research year. Also, the Adenoviral vectors with reporter genes of tk and luciferase were constructed. The luciferase gene expression in live mouse model was non-invasively imaged and the result was posted in 2003 Annual Meeting of ASGT (American Society of Gene Therapy).

15. SUBJECT TERMS
prostate cancer, gene therapy, imaging

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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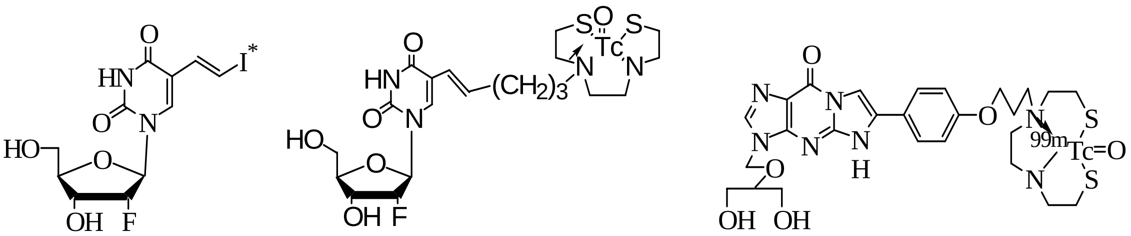
Introduction

The objective of this project is to develop a noninvasive imaging assay using single photon emission computed tomography (SPECT) for assessment of gene therapeutic efficacy and diagnosis of metastasis of prostate cancer.

Currently, nuclear imaging technology has demonstrated the greatest potential to noninvasive image gene activity in animals and humans due to its high sensitivity. By replacing the acyclovir (ACV) with a radioactive analogue it is possible to noninvasively and repeatedly monitor the *in vivo* distribution of the transduced tk construct. As a result, it may assist in determining the optimal timing for ACV administration, confirming the cytotoxic sites, and assessing the therapeutic efficacy. Further refinement of this technology may also provide a non-invasive approach in identifying any metastasis sites in a clinical setting.



IVFRU FTcAU Tc-99m-TGCV

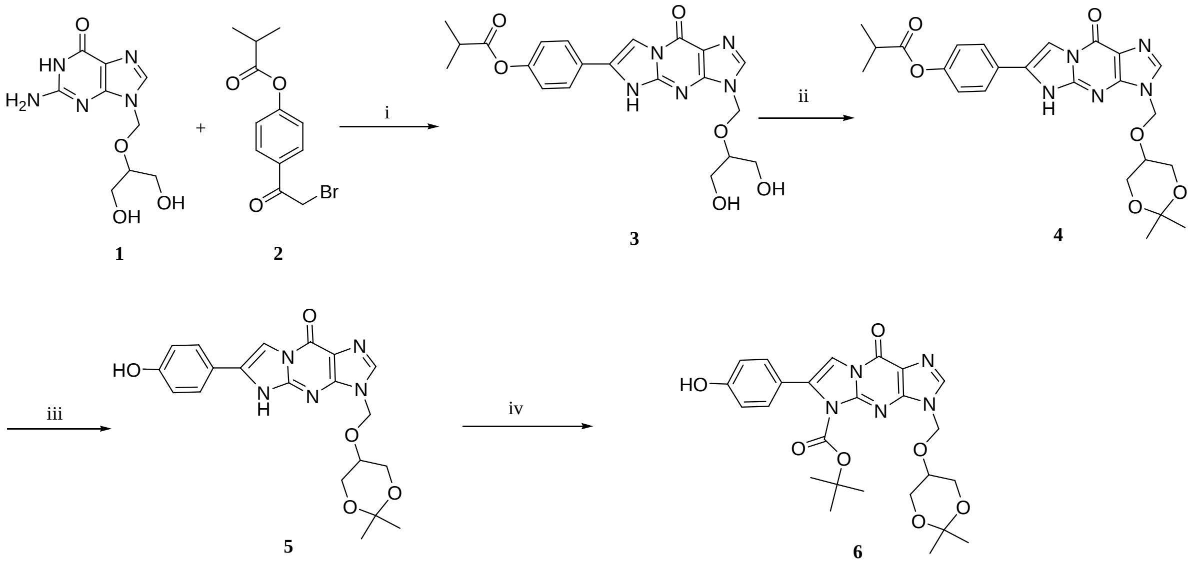
In the original plan we proposed to synthesize a novel thymidine kinase (TK) substrate, I-123 labeled 1-(2-deoxy-2-fluoro-β-D-ribofuranosyl)-5(E)-(2-iodovinyl)uracil (IVFRU). However, the progress of Tc-99m chemistry that Tc-99m labeled radiopharmaceuticals, such as TRODAT, being able to penetrate lipid membrane raises our interest to synthesize a Tc-99m labeled TK substrate for the purpose due to the nearly optimal nuclear properties of Tc-99m, as well as its availability and low cost. As a result we modified our plan by switching the originally proposed I-123 labeled IVFRU with Tc-99m labeled TK substrates. After the first report of synthesis of FTcAU in 2004, we have synthesized another potential TK substrate, Tc-99m-TGCV and submitted a manuscript to the Angewandte Chemistry by last December.

The gene directed enzyme prodrug treatment approach involves prodrug-metabolizing-gene delivery, followed by systemic administration of its specific prodrug. In this study the prostate tumor specific cytotoxic gene expression is realized by a PSA-specific promoter driven thymidine kinase gene vector, AdPSA-tk. The PSA promoter has demonstrated the utility for tissue-specific toxic gene therapy for prostate cancer(Gotoh A, Ko SC, Shirakawa T, et al. 1998 Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. J Urol 160:220-9). But the expression of naïve PSA and the foreign gene overexpression elicited by PSA promoter has not been well determined in normal animals although it's an important issue for therapy safety. Using a small animal optical imaging device we have demonstrated that the AdPSA-Luc can generate high level expression of luciferase gene under the control of the 5837 bp long PSA promoter in lungs of normal mice via tail vein injection. To our knowledge this is the first report that unequivocally demonstrates specific gene expression in lung tissue elicited by a PSA promoter. This may predict PSA expression in lungs of normal mice. These results indicate the potential limitations of the suicide gene therapy of prostate cancer based on the selectivity of PSA promoter. By contrary, it has encouraging implications for the further development of vectors via PSA to enable gene therapy for pulmonary vascular diseases.

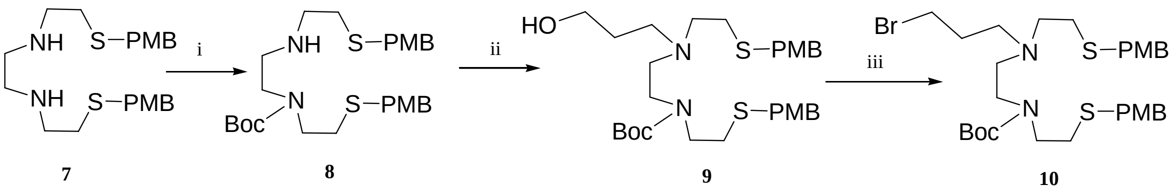
Body

1. *Chemistry*

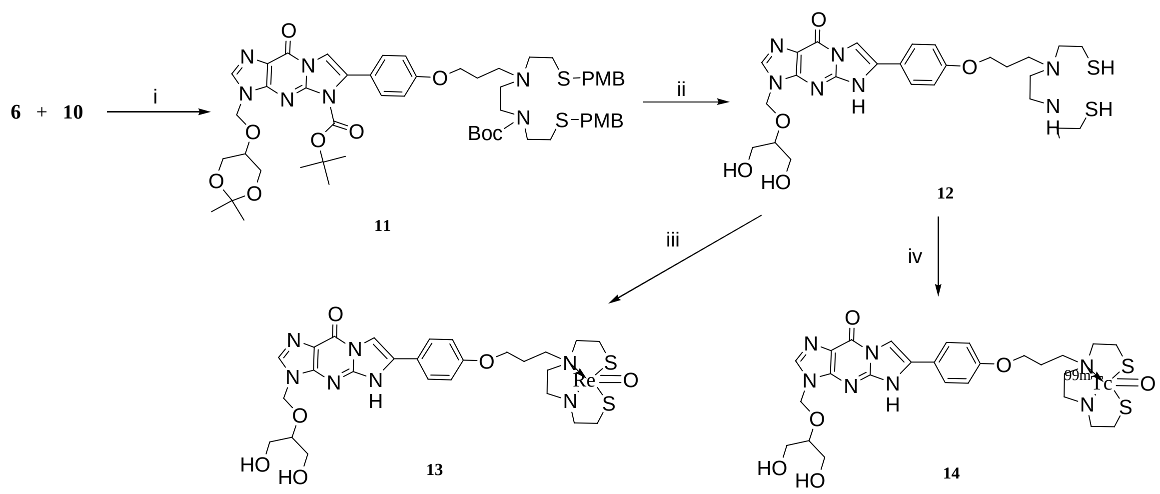
Synthesis of the target molecule, 3-((1,3-dihydroxypropan-2-yloxy)methyl)-6-(4-(3-((2-mercaptoethyl)(2-(2-mercaptoethylamino)ethyl)amino)propoxy)phenyl)-3H-imidazo[1,2-f]purin-9(5H)-one [Tc-99m] **14** (Tc99m-TGCV), was started from gancyclovir **1**. The synthesis is briefly described as following schemes.



Scheme 1. Reagents: (i)NaH/DMF; ii) DMF, 2-methoxypropene, TsOH•H_2O; (iii) NH_3-MeOH; (iv) Boc_2O, 1,4-dioxane, aq Na_2CO_3.



Scheme 2. Reagents: (i)Boc_2O, 1,4-dioxane, aq Na_2CO_3; (ii) acetonitrile, 3-bromo-1-propanol, DIEA; (iii)NBS, PPh_3, DMF.



Scheme 3. Reagents: (i) $CHCl_3$, aq Na_2CO_3, $Bu_4N^+Br^-$; (ii) $Hg(OAc)_2$/TFA, H_2S; (iii) $(Bu_4N)^{+(}ReOCl_4)^-$. (iv) [^{99m}Tc]$NaTcO_4$, Sn-gluceptate

2. *Biology*

We constructed an adenovirus vector (AdPSA-Luc), containing firefly luciferase gene under the control of the 5837 bp long prostate-specific antigen promoter. A charge coupled device (CCD) video camera was used to noninvasively image expression of firefly luciferase in nude mice on days 3, 7, 11 after injection of $2x10^9$ PFU of AdPSA-Luc virus via tail vein. The result showed highly specific expression of the luciferase gene in lungs of mice from day 7 while AdCMV-Luc, containing a CMV promoter and luciferase gene, just led to high level of luciferase expression in livers. The finding indicates the disadvantage of the suicide gene therapy of prostate cancer based on selectivity of PSA promoter. By contrary, it has encouraging implications for further development of vectors via PSA promoter to enable gene therapy for pulmonary diseases.

Key Research Accomplishments:

Chemistry: The Tc-99m labeled TGCV analog, 3-((1,3-dihydroxypropan-2-yloxy)methyl)-6-(4-(3-((2-mercaptoethyl)(2-(2-mercaptoethylamino)ethyl)amino)propoxy)phenyl)-3H-imidazo[1,2-f]purin-9(5H)-one [Tc-99m], was successfully synthesized.

Biology: In the first we demonstrated that the AdPSA-Luc can generate high level expression of luciferase gene under the control of the 5837 bp long PSA promoter in lungs of normal mice via tail vein injection.

Reportable Outcomes:

Chemistry: A report of synthesis of a potential Tc-99m labeled TK substrate, Tc-99m-TGCV, was submitted to *Angewandte Chemistry*;

Biology: Highly specific expression of luciferase gene in lungs of naïve nude mice directed by prostate-specific antigen promoter was reported in *Biochemical and Biophysical Research Communications.*

Conclusions:

1. We have obtained a Tc-99m labeled GCV analog and evaluation of its activity as a TK substrate is undergoing.
2. PSA expression in lungs of normal mice indicate the potential limitations of the PSA promoter mediated suicide gene therapy of prostate cancer.

Reference:

1. Synthesis of a technetium-99m labelled tricyclic gancyclovir analog for non-invasive reporter gene expression imaging, Y. Zhang, J. Lin, and D. Pan, *Angewandte Chemistry*, submitted
2. Highly specific expression of luciferase gene in lungs of naïve nude mice directed by prostate-specific antigen promoter, H. Li, J. Li, G. Helm and D. Pan, *Biochemical and Biophysical Research Communications*, 2005, 334(4), 1287-1291.
3. Synthesis of a novel Tc-99m labeled TK repot probe, 2'-Deoxy-2'fluoro-5-{3-oxo[*N*,*N*-bis(2-mercaptoethyl)ethylenediaminato][Tc-99m] technetium(V)-1(*E*)-propenyl}uridine, Y, Zhang, X. Dai, D. Kallmes and D. Pan, *Tetrahedron Letters*, 2004, 45, 8673-8676.
4. Highly Specific Expression of the Luciferase Gene in Lungs of Naïve Nude Mice Directed by Prostate Specific Antigen Promoter, Hongwei Li, Jin Zhong Li, Gregory A. Helm, Dongfeng Pan, *The 8th Annual Meeting of the American Society of Gene Therapy,* June 1-5, 2005, St. Louis, MO.

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Biochemical and Biophysical Research Communications 334 (2005) 1287–1291
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Highly specific expression of luciferase gene in lungs of naïve nude mice directed by prostate-specific antigen promoter

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Received 5 July 2005
Available online 18 July 2005

Abstract

PSA promoter has been demonstrated the utility for tissue-specific toxic gene therapy in prostate cancer models. Characterization of foreign gene overexpression in normal animals elicited by PSA promoter should help evaluate therapy safety. Here we constructed an adenovirus vector (AdPSA-Luc), containing firefly luciferase gene under the control of the 5837 bp long prostate-specific antigen promoter. A charge coupled device video camera was used to non-invasively image expression of firefly luciferase in nude mice on days 3, 7, 11 after injection of 2×10^9 PFU of AdPSA-Luc virus via tail vein. The result showed highly specific expression of the luciferase gene in lungs of mice from day 7. The finding indicates the potential limitations of the suicide gene therapy of prostate cancer based on selectivity of PSA promoter. By contrary, it has encouraging implications for further development of vectors via PSA promoter to enable gene therapy for pulmonary diseases.

Keywords: Prostate-specific antigen promoter; Non-invasive imaging; Specific expression; Lungs; Naïve nude mice; Prostate cancer; Gene therapy; Adenovirus; Luciferase; Pulmonary diseases

Prostate cancer is the most common malignancy diagnosed and the second leading cause of cancer death in men in the United States [1]. Principal treatments in patients with advanced prostate cancer are based on androgen ablation strategies but most such patients tends to have hormone refractory disease later in the course of treatment [2]. Recent advances in molecular biology offer new hope in the form of gene therapy. A popular cytotoxic gene therapy approach is gene-directed enzyme prodrug gene therapy, also known as suicide gene therapy [3,4]. Its aim is to avoid systemic toxicity, due to the lack of tumor specificity of existing cytotoxic agents, by ensuring that they are generated in high concentrations only at the tumor site, thereby, increasing the therapeutic index [5]. Selectivity and efficacy are the most critical factors in such successful suicide gene therapy.

In that context, tumor- or tissue-specific promoters have been used to target tumor cells selectively. Prostate-specific antigen (PSA) is expressed primarily in both normal prostate epithelium and the vast majority of prostate cancers. Increase in serum PSA during endocrine therapy are generally considered as evidence for prostate cancer recurrence or progression to androgen independence [6]. Target-specific expression of therapeutic toxic genes in tumor cells through the use of tissue-specific promoters could decrease their toxic effect on neighboring normal cells, when virus-mediated gene delivery results in their infection. The PSA promoter has been demonstrated the utility for tissue-specific toxic gene therapy for prostate cancer [7]. But the expression of naïve PSA and the foreign gene overexpression elicited by PSA promoter has not well determined in normal animals although it is an important issue for therapy safety.

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0006-291X/$ - see front matter
doi:10.1016/j.bbrc.2005.07.025

The cooled CCD optical imaging belongs to a new generation of in vivo imaging technologies that use fluorescent or bioluminescent reporter genes to produce a signal from within a living animal. The CCD approach detects low levels of luminescence consistently and reproducibly from fur-covered animals without the need for an external light source [8,9]. CCD camera has been used to non-invasively image luciferase-expressing human prostate tumors and metastases in nude mice [10]. Here we employed the CCD imaging system to image luciferase expression in normal nude mice after tail-vein injection of a prostate-specific adenovirus vector (Ad-PSA-Luc).

Materials and methods

Cell lines and cell cultures. C4-2 was established from LNCaP tumors propagated in castrated hosts [11,12], and WH was established from a human bladder transitional cell carcinoma [13]. All cell lines were maintained in T-medium (Invitrogen, Carlsbad, CA), with 5% fetal bovine serum (FBS; Invitrogen).

Construction of recombinant adenoviruses. All plasmid constructs were prepared using standard methods [14]. The original pPSA-Luc containing the 5.8-kb PSA promoter was obtained from Dr. Leland W.K. Chung. It was generated by inserting a *Hin*dIII fragment of the PSA promoter in the multiple cloning site (MCS) of pGL3-basic vector (Promega, Madison, WI). pShuttle-PSA-Luc was generated by inserting a *Kpn*I/*Sal*I fragment of pPSA-Luc, which contains PSA promoter, luciferase gene, and SV40 late poly(A) signal, in the MCS of pShuttle vector [15].

AdEasy Vector System [15] was used for construction of recombinant adenovirus of the firefly luciferase under the control of the 5837 bp prostate-specific antigen promoter. Briefly, pShuttle-PSA-Luc was linearized with *Pme*I and co-transformed into *Escherichia coli* strain BJ5183 together with pAdeasy-1, the viral DNA plasmid. The pAdeasy-1 is E1 and E3 deleted, its E1 function can be complemented in 293A cells. The recombinant adenoviral construct, which was named pAd-PSA-Luc was then cleaved with PacI to expose its inverted terminal repeats (ITR) and transfected into 293A cells to produce viral particles. The recombinant virus was identified with restriction analysis, PCR, RT-PCR, and detection of luciferase activity. The recombinant virus Ad-PSA-Luc was purified through two cesium chloride gradients, and then purified virus was desalted by dialysis at 4 °C against 10 mM Tris–hydrochloric acid buffer with 10% glycerol and stored in aliquots in liquid nitrogen. The titer of virus preparations was determined by plaque assay according to the application manual of the pAdEasy Vector System.

As a control, AdCMV-Luc, which contains CMV promoter and luciferase gene, was constructed as described above.

Evaluation of expression of AdPSA-Luc in prostate and non-prostate cancer cells. Cells C4-2 or WH at ~80% confluency in 24-well plates were infected with AdPSA-Luc or AdCMV-Luc at the specified PFU per cell. From 24 h after transduction, an aqueous solution of substrate, D-firefly lucifern (Xenogen, Alameda, CA) was added into cells (5 μl/well, 15 mg/ml) and the imaging was performed by an IVIS CCD camera (Xenogen) [8].

In vivo gene transfer. Six- to eight-week-old male athymic nude mice (nu/nu strain [Balb/c background], Harlan Sprague–Dawley, Indianapolis, IN) were used for all in vivo experiments. They were kept under pathogen-free conditions in laminar flow boxes in accordance with established institutional guidelines and approved protocols. Adenoviruses (2.0×10^9 PFU) AdPSA-Luc and AdCMV-Luc were injected into naïve nude mice ($n = 4$) , respectively, via tail vein.

CCD imaging to detect in vivo luciferase expression. On days 3, 7, and 11, the CCD images were obtained using a cooled IVIS CCD camera (Xenogen) and images were analyzed [9]. Light was monitored in all of the experiments described at 5 min after injection of luciferin. The CCD signals were quantified as total relative light units per minute of acquisition time (RLU/min) in the region of interest (ROI). On day 11, mice were sacrificed and isolated organs were imaged.

Results and discussion

Tissue-specific expression of AdPSA-Luc in human cancer cell line

We constructed an adenovirus vector (AdPSA-Luc), which can express firefly luciferase under the control of the 5837 bp prostate-specific antigen promoter (Fig. 1).

The tissue-specific expression of AdPSA-Luc was tested by CCD camera imaging in human prostate cancer PSA-producing cell line C4-2, an androgen-independent subline of LNCaP, and non-prostate cancer cell line WH. Fig. 2A shows that AdPSA-Luc led to significantly higher level of luciferase expression in C4-2 than in WH on days 2 after transduction when they were transduced at 80, 40, 20, 10, 5, and 2.5 PFU of AdPSA-Luc per cell. We imaged the infected cells from 2 to 5 days after transduction and the similar results were obtained. The infectivities of C4-2 and WH were compared by using the same range of AdCMV-Luc as the infecting viruses (Fig. 2B).

The high tissue-specificity of the long PSA promoter has been well determined in its recombinant plasmids, transgenic animals as well as adenoviral vectors [7,16,17]. Our results are consistent with these publications. In contrast, Shi et al. [18] reported that the PSA promoter cassette in helper-dependent adenoviral

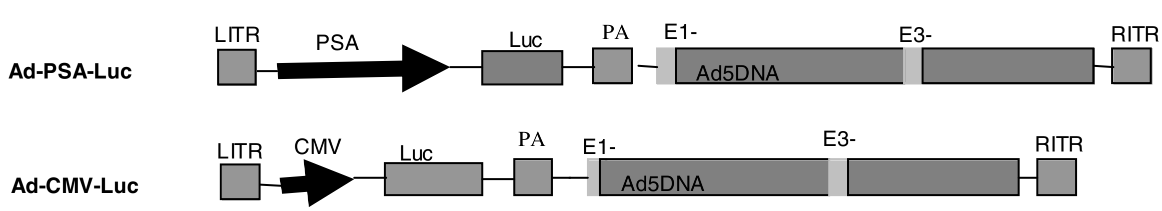

Fig. 1. Schematic representation of Ad vectors used in this study. ITR, Ad inverted terminal repeats; CMV, human cytomegalovirus promoter; PSA, prostate specific antigen promoter; Luc, firefly luciferase gene.

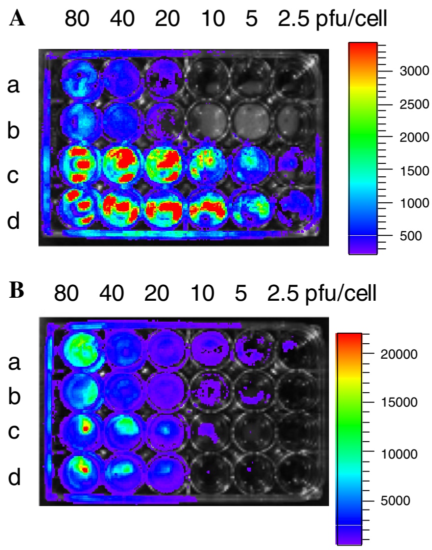

Fig. 2. Comparison of luciferase expression following transduction of prostate and non-prostate cell line with AdPSA-Luc. The PSA-positive cell line C4-2 (c,d) and non-prostate cell line WH (a,b) were transduced with AdPSA-Lul (A) or AdCMV-Luc (B) and imaged by CCD camera 2 days later. The acquisition times: (A) 5 min; (B) 10 s.

vectors maintained strict tissue-specific expression, but lost specificity when expressed from first-generation adenoviral vectors. The difference amongst these results may be due to variations in sequence of the promoters or difference in methodology. In addition, we observed that AdPSA-Luc just elicited higher level of Luc expression in C4-2 cells than in WH cells in a short period (24 h) after transduction beyond 200 PFU per cell and decreased rapidly Luc expression in C4-2 cells into an extent even less than in WH cells because of higher adenoviral toxicity in C4-2.

Ad-PSA-Luc elicited highly specific expression of luciferase gene in lungs of normal nude mice

A CCD video camera was used to non-invasively image expression of luciferase in nude mice ($n = 4$) on days 3, 7, and 11 after injection of 2×10^9 PFU of AdPSA-Luc virus via tail vein. The CCD signals were quantified as total relative light units per minute of acquisition time (RLU/min). On day 3, only weak liver and chest signals can be detected. On day 7, light signals in the chests and lower abdomens were apparent. The chest signals were emitted by 1.8×10^3, 1.2×10^4, and 6.0×10^4 RLU/min respectively from day 3 to 11 (Figs. 3A–D). On day 7, the outlines of lungs were imaged distinctly (Fig. 3B). As a control, AdCMV-Luc containing CMV promoter and luciferase gene was injected into normal nude mice via tail vein. The strong liver signals (5.3×10^6,

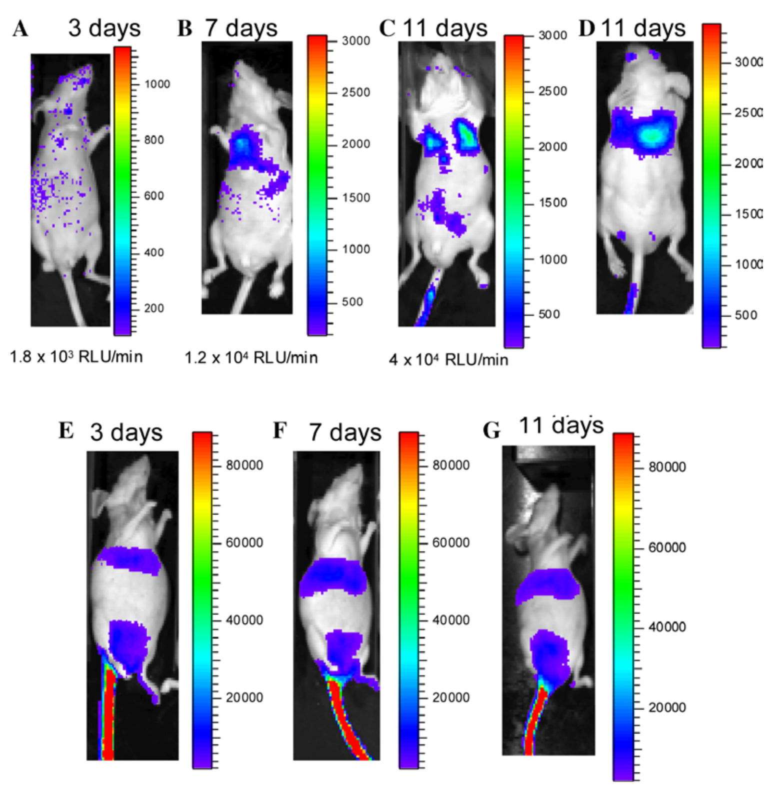

Fig. 3. Adenoviral vector-mediated luciferase gene delivery and expression in the lung and liver after systemic administration. CCD images of mice injected with either the prostate-specific AdPSA-Luc (A–D) or AdCMV-Luc (E–G) via tail vein. The images represent the results from one animal of each cohort at 3, 7, and 11 days post-injection. The relative light intensity emitted from the animal was quantified by image analysis software and represented by the color scale, shown next to the images. The acquisition times: (A–D) 5 min; (E) 30 s; and (F,G) 5 s. (D) The mouse was imaged from the back. The acquisition times were reduced to offset saturated liver signal intensities in AdCMV-Luc cohort.

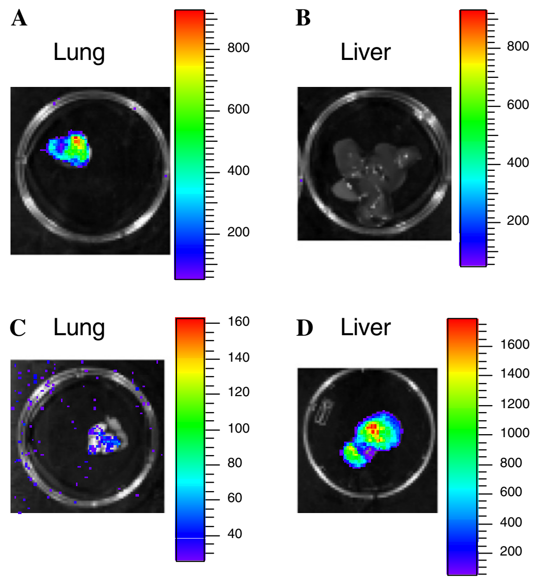

Fig. 4. Imaging of the isolated organs from mice in Fig. 3. (A,B) The lung and liver from the AdPSA-Luc injected mouse; (C,D) the lung and liver from the AdCMV-Luc injected mouse. The acquisition times: (A–C) 5 min; (D) 5 s.

3.6×10^7, and 4.1×10^7 RLU/min, respectively) could be seen from 3 to 11 days after injection (Figs. 3E–G). On day 11, mice were sacrificed and isolated organs were imaged. The signals in the chest injected with AdPSA-Luc were found to originate from lungs (Fig. 4). The signal in whole lung from AdPSA-Luc injected mice displayed about 190-fold higher than from AdCMV-Luc injected mice (Fig. 4). However, the signals in the isolated livers of the mice injected with AdCMV-Luc appeared to be $\sim 1.0 \times 10^5$-fold higher than in the lungs while the signals could not be detected in the isolated livers of the mice injected with AdPSA-Luc (Fig. 4). According to these results, AdPSA-Luc displayed high level of specific expression in lungs compared to AdCMV-Luc.

Gene-directed enzyme prodrug treatment approach involves prodrug metabolizing-gene delivery, followed by systemic administration of its specific prodrug. This activated drug not only kills cells that produced the toxic drug, but also neighboring cancer cells, an effect called the bystander effect [19]. The use of tumor or tissue-specific promoter systems in the context of this therapy makes sense because it further enhances tumor or tissue-specific expression of the suicide gene and therefore therapy safety. Because prostate is a non-essential organ, such suicide gene therapy can be targeted with tissue-specific rather than tumor-specific promoters. Prostate is known to have several specific tissue expressed proteins. Of these proteins PSA is considered the most important marker for prostate cancer. Its promoter and enhancer regions were recently identified and used in several gene therapy experiments [7,16,20]. A prostate-specific adenovirus vector and a CCD camera imaging system have been employed successfully on the detection of lung metastasis in a human-prostate cancer model [10]. But the gene expression elicited by PSA promoter has not well determined in other organs or tissues of normal animals except for prostate.

We have reported here that the significant luciferase expression was detected in lungs of normal nude mice with a CCD camera by systemic tail-vein injection of AdPSA-Luc. Moreover, AdPSA-Luc led to 190-fold higher luciferase expression in the lungs than AdCMV-Luc did. Selectivity and efficacy are the most critical factors in successful suicide gene therapy. Since AdPSA-Luc demonstrates high level expression of linked gene luciferase expression in the lungs of normal animals, enzyme based suicide gene therapy for prostate cancer, based on the selectivity of a PSA promoter, should be characterized more carefully for the safety concern.

PSA belongs to the human kallikrein gene family of serine proteases and has been characterized as a "prostate-specific" antigen [21,22], but it has been demonstrated that PSA is not only a prostate-specific kallikrein, but it is also expressed in other tissues. For example, the expression of PSA mRNA in human pituitary gland and uterus has been verified by RT-PCR [23]. PSA expression has been shown in the lung tissue from a female patient with lung adenocarcinoma, in which PSA immunoreactivity was localized by immunohistochemistry to normal epithelial cells adjacent to the tumor which was completely negative for PSA [24]. PSA is also expressed in normal human embryonic lung cells, WI-38 [25]. In this study, we demonstrated that AdPSA-Luc elicited high expression level of linked luciferase gene in lungs of normal mice. This may indicate the possible expression of PSA in lungs of normal nude mice.

The promise of gene therapy for health care will not be realized until gene delivery systems are capable of achieving efficient, cell-specific gene delivery in vivo [26]. Ad-mediated gene transfer has limitations in vivo application and their potential utility is compromised by their restricted tropism, despite the promising features of Adenoviral vectors, such as in vivo stability and efficiency [27]. Administration of AdPSA-Luc via tail vein injection into mice resulted in high level specific expression of luciferase transgene in the lungs not in the livers, compared to AdCMV-Luc, which mainly elicited luciferase expression in the livers. This may predict that gene delivery and expression via PSA promoter will offer new gene therapeutic opportunities in pulmonary diseases such as pulmonary hypertension, pulmonary thromboembolic disease, and pulmonary carcinoma.

In summary, we demonstrated that the AdPSA-Luc can generate high level expression of luciferase gene under the control of the 5837 bp long PSA promoter in lungs of normal mice via tail vein injection. To our knowledge, this is the first report that unequivocally

demonstrates specific gene expression in lung tissue elicited by a PSA promoter. This may predict PSA expression in lungs of normal mice. These results indicate the potential limitations of the suicide gene therapy of prostate cancer based on the selectivity of PSA promoter. By contrary, it has encouraging implications for the further development of vectors via PSA to enable gene therapy for pulmonary vascular diseases.

Acknowledgments

We thank Dr. Leland W.K. Chung for helpful advice and materials support. The research was financially supported by DOD Grant PC010112.

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